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Acute stress alters the rates of degradation of cardiac muscle proteins

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ABSTRACT

Stressful experiences can have detrimental effects on many aspects of health and wellbeing. The zebrafish (*Danio rerio*) is a widely used model for stress research and a stress phenotype can be induced by manipulating the environmental conditions and social interactions. In this study we have combined a zebrafish stress model with the measurement of degradation rates of soluble cardiac muscle proteins. The results showed that the greater the stress response in the zebrafish the lower the level of overall protein degradation. On comparing the rates of degradation for individual proteins it was found that four main pathways were altered in response to stress conditions with decreased degradation for proteins involved in glucose metabolism, gluconeogenesis, the ubiquitin-proteasome system (UPS) and peroxisomal proliferator-activated receptor (PPAR) signalling pathways. Taken together, these data indicate that under stress conditions zebrafish preserve cardiac muscle proteins required for the 'fight or flight' response together with proteins that play a role in stress mitigation.

Significance: This study is the first to investigate the impact of stressful experiences on the dynamics of protein turnover in cardiac muscle. Using an established zebrafish model of human stress it has been possible to map key pathways at the protein level. The results show that the rates of degradation of cardiac proteins involved in glucose metabolism, UPS activity, hypoxia and PPAR signalling are decreased in stressed zebrafish. These findings indicate that proteins involved in the 'fight or flight' response to stress are conserved by the heart together with proteins that play a role in stress mitigation. This work provides the basis for more detailed investigations aimed at understanding the molecular effects of stress, which has implications for human health and disease.

1. Introduction

Stress is a composite, multidimensional concept that has complex physiological as well as behavioral effects. A universal definition of stress remains elusive but can be considered as the biological response elicited by an organism to a perceived threat to its homeostasis. The activation of stress pathways is an initial adaptive response and whilst this can be beneficial, increasing survival chances, intense acute or chronic stress may lead to maladaptive responses that are detrimental in the long term [1]. Indeed, a normal stress response is required for healthy living but persistent stressful experiences can manifest in a range of physical and psychological conditions [2,3].

The zebrafish (*Danio rerio*) is an established model organism in biomedical research, its small size, low maintenance and fast reproduction having the advantage of reducing both the time and cost of experimental programmes. The zebrafish is increasingly being employed in studies that are focussed on understanding the biological mechanisms of human stress and stress-related disorders [4–8]. However, like any model, the zebrafish has some limitations. There are distinct differences between zebrafish and humans, for example, zebrafish live in an aquatic environment, have a duplicated genome and certain areas of the brain are less developed [9]. Nonetheless, there are strong parallels in terms of the organisation and function of the stress response systems. In particular the hypothalamic-pituitary-adrenal (HPA) axis in humans and its analogue the hypothalamic-pituitary-interrenal (HPI)

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axis in zebrafish are evolutionarily conserved and share extensive homologies [10,11].

Stress responses can be generated in zebrafish by a range of stimuli that include physical stressors (e.g. capture and confinement), environmental stressors (e.g. changes in water temperature) and psychological stressors (e.g. predator cues and overcrowding). The stress response in fish involves a complex cascade of biochemical and physiological changes that can be broadly categorised into primary, secondary and tertiary responses [12]. In the primary response the perception of an altered state results in the release of catecholamines (adrenaline and noradrenaline) and corticosteroids (cortisol) [13,14]. In the secondary response various metabolic pathways are activated leading to the mobilization of glycogen reserves and an elevation of circulating glucose that serves as a substrate to meet increased energy demands from tissues. Similarly, there are adjustments to haematological factors and hydromineral balance [15]. The tertiary response represents the impact on whole animal performance characteristics and modification of behaviour [16].

Stressful conditions also elicit a generalised response at the level of the cell. A well-defined feature of this response is the production of heat shock proteins (HSPs), a family of molecular chaperones that play an important role in protein refolding and preventing protein aggregation [17]. Another key process during cellular stress is protein degradation. One of the primary pathways of intracellular protein degradation, the ubiquitin-proteasome system (UPS) has been implicated in sensing, signalling and mediating the stress response [18]. By either promoting or inhibiting the degradation of proteins the cell can rapidly maintain essential functions and limit non-critical activities.

Researchers are now beginning to utilise proteomics as a tool to investigate the molecular responses to stress in zebrafish models. Magdeldin and colleagues [19] showed that elevated levels of anxiety in zebrafish produces an up-regulation of HSPs, metabolic enzymes and cation transporters along with structural proteins associated with locomotive activity. Further, differential expression of proteins involved in mitochondrial function, hypoxia and oxidative stress has been observed in the brains of zebrafish subjected to chronic unpredictable stress (CUS) [20]. The heart is also susceptible to stress and recently it has been reported that exposure to short periods of stress can impair the natural capacity of heart regeneration in the zebrafish [21]. We have previously developed an advanced proteomics methodology to measure the absolute rates of protein turnover in the zebrafish heart, which involves the dietary administration of a stable isotope-labelled amino acid and subsequent analysis of protein populations by high resolution LC-MS/MS [22]. In the current study we have applied this approach to determine the effect of acute stress on the degradation of zebrafish cardiac muscle proteins. Our findings have enabled changes to pathways resulting from stress responses to be mapped at the protein level.

2. Materials and methods

2.1. Experimental conditions

Zebrafish were maintained in the University of Liverpool aquarium at 28 °C ± 1 °C (at pH7.6) on a 12 h light:12 h dark photoperiod throughout the study. Zebrafish were housed in identical 25 cm × 11 cm × 15 cm zebrafish tanks (Pentair Aquatic Ecosystems, Manchester, UK). Daily checks were carried out for pH, ammonia, nitrite and nitrate levels, with desired levels being pH7.6, 0 mg/l, 0 mg/l and < 20 mg/l respectively. Weekly water changes replaced at least 20% of the water of the system.

For stress studies, zebrafish were split into three study groups (n = 10 per condition): barren and grouped and enriched. The barren fish were kept in individual tanks where they could not see any other fish and the tanks were otherwise empty. The grouped fish were all kept in a single tank with gravel and plant life as a group. The enriched fish were kept in individual tanks where they had the ability to see other fish. The tanks also contained synthetic gravel (> 5 mm) and plant life. One plant type ("submerged plant") simulated broad-leaved bright green submerged and rooted aquatic vegetation with a black resin root base, while the other ("overhanging plant", green Supa Fern®) had no root base, resembling overhanging ferns or similar unrooted fine-leaved vegetation to provide overhead cover. The temperature, light cycle and feeding rate were kept constant throughout the experiment. The fish were observed at least three times daily, at morning and afternoon feeds then at approximately 5 pm. Fish from all of the experimental groups were fed a diet in which 50% of the L-leucine (that proportion added as crystalline amino acid) was replaced with [$^2H_7$]-L-leucine (98% purity) (Cambridge Isotope Laboratories, Andover, MA, USA) [22] for six weeks prior to the commencement of the study. At the experimental start (t = 0) the diet was changed to an unlabelled diet and five fish immediately sacrificed weighed and dissected. After two weeks of stress exposure, five additional fish from each study group were sacrificed. No fish sustained injuries during the behavioral assays. Fish were killed in accordance with UK Home Office Schedule One regulations. This research was approved by the Ethics Committee at the University of Liverpool.

2.2. Measurement of cortisol in tank water

Water was removed from three of the tanks used for the barren and enriched fish and the single tank for the group of fish. Particulate matter removed prior to loading onto a SepPak solid phase extraction cartridge (Sep-Pak C18, 500 mg sorbent per cartridge, Waters, Manchester, UK) using a reservoir attachment. The cortisol fraction was eluted with 2 ml methanol at a flow rate of 1 ml/min. The eluate was then concentrated under vacuum using a MiVac Concentrator (Genevac, Ipswich, UK) to a volume of approximately 300 µl.

The concentration of the cortisol was measured using an enzyme-linked immunosorbant assay (ELISA) (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, 100 µl of each sample were pipetted into individual wells of a 96-well plate and 50 µl of assay buffer (Tris-buffered saline) added. Alkaline phosphatase conjugated to cortisol (50 µl) was aliquoted into each well, followed by 50 µl of a mouse monoclonal anti-cortisol antibody. The plate was incubated at room temperature for 2 h after which the contents of were discarded and each well washed with 400 µl of wash solution (Tris-buffered saline plus detergent). 200 µl of the substrate solution *p*-nitrophenyl phosphate in buffer) were added and the plate incubated at room temperature for 1 h. 50 µl of trisodium phosphate in water were then added to each well. The optical density was measured at 405 nm using a Varioskan plate reader (Thermo, Hemel Hempstead, UK) and the concentration of cortisol determined from a standard curve. The statistical difference of cortisol concentrations between barren and enriched groups was determined using a student's *t*-test.

2.3. Zebrafish heart preparation

Immediately after sacrifice, fish were weighed and the heart of each fish was dissected within approximately 10 s. The heart was then rinsed in ice cold phosphate buffered saline then placed in an Eppendorf tube and stored at −80 °C until analysed. The heart samples (approximately 1 mg wet weight of tissue) were mechanically homogenised in 500 µl of 1× phosphate buffered saline (Invitrogen, Carlsbad, United States) containing Complete Protease Inhibitors (Roche, Lewes, UK). The homogenate was centrifuged at 19,000 × *g* at 4 °C for

45 min and the supernatant collected. The protein concentration of the supernatant was determined using the Coomassie Plus Protein Assay (Pierce Biotechnology, Rockford, IL, USA).

2.4. 1-D SDS-PAGE

The soluble proteins (20 μg) from zebrafish heart were separated by 1-D SDS-PAGE using a Mini Protean Tetra system (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Samples were electrophoresed at a constant potential of 200 V through a 15% w/v polyacrylamide resolving gel with a 4% w/v stacking gel. Samples were incubated at 95 °C for 5 min in a reducing buffer (125 mM Tris-HCl; 140 mM SDS; 20% v/v glycerol; 200 mM DTT and 30 mM bromophenol blue) prior to loading. Gels were stained with Coomassie Blue (Bio-Rad).

2.5. In-gel digestion

Gel lanes were cut into 12 slices and each slice placed in distilled deionised water (50 μl). The water was then removed and the gel piece was treated with destain solution (10 μl of ACN/100 mM ammonium bicarbonate 1,1 v/v). The protein disulphide bonds were reduced by the addition of dithiothreitol (20 μl of 10 mM for 30 min) and alkylated by iodoacetamide (20 μl of 55 mM for 30 min incubation in the dark). Each gel slice was dehydrated in acetonitrile. Trypsin (Roche) (0.2 μg/μl in 50 mM acetic acid) was added at a ratio of protein:trypsin 50:1 and the digestion allowed to proceed overnight at 37 °C. The peptides were then extracted from the gel by addition of acetonitrile and then dried under vacuum in a MiVac concentrator (Genevac, Ipswich, United Kingdom) prior to resuspension in 50% methanol.

2.6. Peptide analysis by LC-MS/MS

LC-MS/MS analysis of peptides was performed in positive ion mode using a Thermo LTQ-Orbitrap XL LC-MS^n mass spectrometer equipped with a nanospray source and interfaced to a Waters nanoAcquity ultra performance liquid chromatography (UPLC) system. The samples (5 μl) were initially desalted and concentrated on a 5 μm Symmetry C18 180 μm × 20 mm trapping column (Waters, Milford, MA, USA). The peptides were then separated on a BEH C18 nanocolumn (1.7 μm, 75 μm × 250 mm, Waters). Mobile phase A comprised 0.1% formic acid in water and mobile phase B comprised 0.1% formic acid in acetonitrile (all Fisher Scientific, Loughborough, UK). A gradient of 10-40% acetonitrile over 120 min was employed with a flow rate of 400 nl/min. Peptides were ionised using a PicoTip emitter (New Objective, Woburn, MA, USA) at 3.5 kV source voltage. Acquisition was in data-dependent mode over the range *m/z* 300-2000 with the top 10 ions being fragmented using the lock mass setting for increased accuracy and comparability. Dynamic exclusion settings allowed a single repeat with a duration of 30 s, keeping a list of 500 ions. Charge state screening was enabled, rejecting unassigned and single positive charge states.

2.7. Proteomic data analysis

Proteomic data were analysed using MaxQUANT with the Andromeda search engine [23,24]. The initial search parameters allowed for two trypsin missed cleavages, carbamidomethyl modification of cysteine residues, oxidation of methionine and acetylation of N-terminal peptides and a false discovery rate (FDR) of 0.01. A mass tolerance of 20 ppm for the precursor ion first search and a tolerance of 6 ppm for main search were allowed along with a fragment mass tolerance of ±0.5 Da. A maximum 1% false discovery rate was used for both protein and peptide identification. Protein identifications were made from a minimum of two peptides per protein including at least one unique peptide. Identified contaminants were removed. An additional parameter, coded as a pseudo post-translational modification was included to search for peptides containing [$^2H_7$] L-leucine.

2.8. Calculation of protein degradation rates

The relative isotope abundance (RIA) of the precursor pool of soluble cardiac muscle was determined using peptides containing two leucine residues from a range of different proteins. These proteins were taken from the list of identified proteins and covered a range of molecular weights. Our previous work has shown good agreement between data derived from di- and tri-leucine peptides As the signal to noise ratio (S/N) for the di-leucine peptides is in general more favourable, we focussed solely on these peptides for determination of RIA. Once the precursor RIA was calculated it was used to deconvolute the peptide ion intensity from mono-leucine peptides to pre-existing "old" protein and newly synthesised protein. This relative partition of intensity over time was calculated over time, allowing the rates of degradation of each protein to be determined. The statistical differences in protein degradation rates between the experimental groups were determined using non-parametric analysis of the data (Mann Whitney for paired analysis, Kruskal-Wallis to compare all three groups).

2.9. Gene ontology cluster analysis

Ontologies from GO, KEGG and Reactome were clustered and statistically evaluated using ClueGO (v2.3.3) and CluePedia (v1.3.3) within the Cytoscape (v3.5.0) environment. Input were UniProt accession numbers, and the species reference database was Zebrafish (ClueGO mapped date 17.11.2016). Thresholding parameters were set to use GO term fusion (hierarchical combined grouping of molecular function, biological process and cellular component), only to include pathways with a statistical significance $p \leq 0.05$, and kappa score 0.3. Term enrichment used a two sided-hypergeometric test including Bonferroni correction for multiple testing. CluePedia was used to associate the genes from the input list to the specific terms. Group comparisons were performed using either "barren" or "grouped" as input for group 1, and "enriched" for group 2.

3. Results

3.1. The stressed phenotype in the zebrafish

In this study three groups of zebrafish were subjected to barren, grouped or enriched conditions in order to elicit a natural stressed phenotype. The response in the different groups was assessed by measuring cortisol in the tank water, which is widely used as an indicator of stress in fish [25–27]. The mean concentration of cortisol was significantly higher in the zebrafish maintained in a barren environment (6938 pg/ml ± 812) compared to the enriched environment (3227 pg/ml ± 504, $p = 0.005$). In the single grouped tank the concentration of cortisol was 4663 pg/ml (Fig. 1). These data indicate that the zebrafish in the barren environment experienced greater stress than the other groups, a finding entirely consistent with previous work that has demonstrated that zebrafish show a strong preference for enriched conditions [28].

3.2. Protein degradation in the zebrafish heart

The zebrafish were maintained for six weeks on the experimental diet containing 50% of leucine in the deuterated form. Throughout the trial all of the zebrafish fed well, remained active and alert. There were

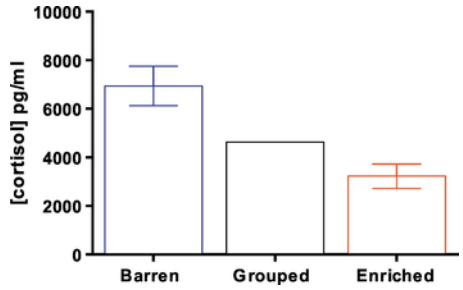

Fig. 1. Measurement of cortisol concentrations. Cortisol was directly measured in water collected from tanks that housed each of the experimental groups of zebrafish. Blue = barren zebrafish; Black = grouped zebrafish; Red = enriched zebrafish. Concentrations in expressed as the mean ± SEM where appropriate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

no fatalities or evidence of abnormality in the fish. Following LC-MS/MS analysis, peptides containing multiple leucine residues were identified and the RIA of the protein precursor pool determined at each time-point using the equations derived in our previous studies [29,30]. The RIA was not found to vary between the different experimental conditions (Fig. 2).

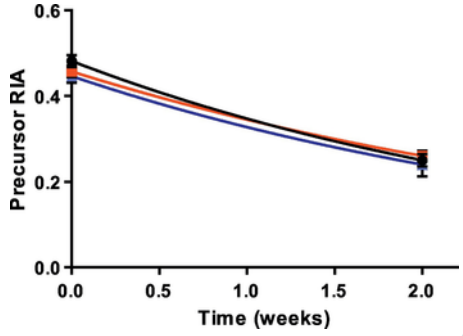

Fig. 2. Calculation of RIA from multi-leucine peptides. The relative abundance of the dietary-supplemented amino acid [$^2H_7$] L-leucine in the precursor pool for protein degradation was determined experimentally. Data were obtained from dileucine containing peptides from multiple proteins across the time-course of the experiment. The relative isotope abundance of labelled leucine in cardiac muscle calculated as determined as 0.46 ± 0.01 decreasing to 0.25 ± 0.01.

The degradation rates of 287 proteins were calculated from two time-points with at least two unique peptides used in the identification (Supplemental Table 1). ClueGO analysis grouped the proteins into fifteen functional families - actin filament depolymerisation, cellular response to oxidative stress, energy derivation by oxidation of organic compounds, gas transport, generation of precursor metabolites and energy, glutathione transferase activity, intermediate filament, one-carbon metabolic process, oxidation-reduction process, oxidoreductase activity-acting on the aldehyde or oxo group of donors, purine ribonucleoside triphosphate metabolic process, response to hypoxia, single-organism catabolic process, small molecule metabolic process and threonine-type endopeptidase activity (Supplemental Fig. 1). This broad range of ontologies is representative of the functions of the soluble cardiac muscle proteome.

The rates of protein degradation across all three conditions ranged from 0 $week^{-1}$ e.g. phosphoenolpyruvate carboxykinase 2, heat shock protein 5 and parvalbumin-2 (barren); ATP synthase subunit beta and phosphoenolpyruvate carboxykinase 2 (enriched) to 8.8 $week^{-1}$ for sterol carrier protein 2B (enriched). The mean rate of protein degradation for the barren cohort was 0.52 $week^{-1}$ ± 0.03 (n = 135 proteins) compared to 0.47 $week^{-1}$ ± 0.02 for the grouped cohort (n = 188) and 0.70 ± 0.06 for the enriched cohort (n = 151). It was possible to calculate the degradation rates for 131 proteins in at least two of the experimental groups and for 57 proteins across all of the conditions (Fig. 4). For these 57 proteins the mean rates of degradation were significantly different (mean k_{deg} barren = 0.54 $week^{-1}$ ± 0.05; mean k_{deg} grouped = 0.47 $week^{-1}$ ± 0.05; mean k_{deg} enriched = 0.68 $week^{-1}$ ± 0.04, n = 57 per group) as shown in Fig. 3. Pairwise comparisons between the barren and enriched conditions also revealed significant differences in the mean rate of protein degradation (Mann Whitney test, p = 0.0022) as did the comparison between the grouped and enriched conditions (Mann Whitney test, p < 0.001). There was no significant difference between the barren and grouped conditions. This indicates that the rate of degradation is decreased in zebrafish exposed to stress environments.

3.3. Comparison of rates of protein degradation of individual cardiac muscle proteins

The degradation rates of individual proteins were compared across the study group (Fig. 5). There were 125 proteins common to both the

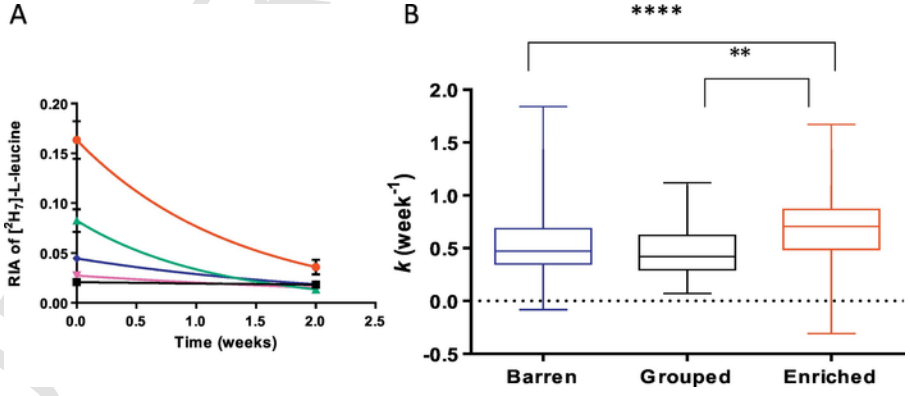

Fig. 3. Comparison of rates of degradation of soluble cardiac proteins in zebrafish maintained in enriched, grouped and barren environments. The degradation rates of 287 proteins were calculated from two time-points with at least two unique peptides used in the identification (A). Proteins shown are Histone H4 (red), heat shock protein 11 (green), proteasome sub-unit β-4 (blue), a-2-HS glycoprotein 2 (pink) and malic enzyme 3 (black). Degradation rates for 57 proteins common to all conditions were compared (B). The intersecting line indicates the median values (k_{deg} barren = 0.47 $week^{-1}$; k_{deg} grouped = 0.42 $week^{-1}$; k_{deg} enriched = 0.71 $week^{-1}$, n = 57 per group). Statistical analysis indicated that there was a difference between the mean rate of protein degradation between the enhanced ("non-stressed") condition and both the barren and grouped ("stressed") conditions. (Kruskal-Wallis test p = 0.0001; mean k_{deg} barren = 0.54 $week^{-1}$ ± 0.05; mean k_{deg} grouped = 0.47 $week^{-1}$ ± 0.05; mean k_{deg} enriched = 0.68 $week^{-1}$ ± 0.04, n = 57 per group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

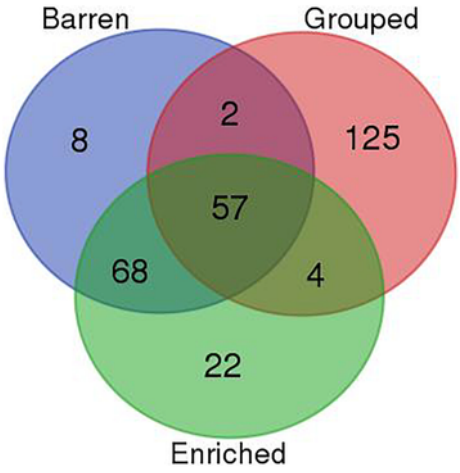

Fig. 4. Overlap of degradation rate constants between experimental groups. The degradation rates of proteins from each experimental condition was determined and compared across the sample set. The commonality between experimental groups is shown.

barren and enriched groups of which 38 showed a decrease in protein degradation in the barren group compared to the enriched. These proteins are involved in ATP metabolic processes, threonine-type endopeptidase activity (proteasome activity) and the peroxisomal proliferator-activated receptor (PPAR) signalling pathway were specifically enriched in those proteins where there was (Fig. 6A). Conversely, 13 proteins showed an increased rate of degradation in the barren cohort compared to the enriched group. GO analysis indicated there was no clustering of these proteins. There were 61 proteins common to both the grouped and enriched conditions with 27 proteins showing a decrease in protein degradation and 5 an increase in protein degradation in the grouped condition compared to the enriched. Those proteins with a decrease in degradation rate were clustered by GO analysis to proteins related to response to hypoxia and nicotinamide nucleotide metabolic processes including glucose metabolism, glycolysis and gluconeogenesis (Fig. 6B). There were 59 proteins common to the barren and grouped cohorts with 24 showing significant changes in protein degradation rate between the groups (13 decreased rates of degradation in the barren group with 11 increased). GO analysis showed no conclusive changes between the cohorts in terms of function of these proteins.

The data were checked manually to determine whether there were changes in the degradation rates of proteins known to be involved in cellular stress responses. There were no significant changes in rate of degradation between the experimental conditions of oxidative enzymes and transport complexes in mitochondria. We also examined HSPs. The rates of protein degradation for nine HSPs were calculated, four of which were determined in each experimental condition. There was only a significant change in the rate of degradation of HSP5, which had a reduced rate of degradation in both the grouped and barren conditions. Indeed, there was no detectable degradation of this protein under the barren condition at all, which is perhaps reflective of the increase in protein concentration observed in a previous study [19].

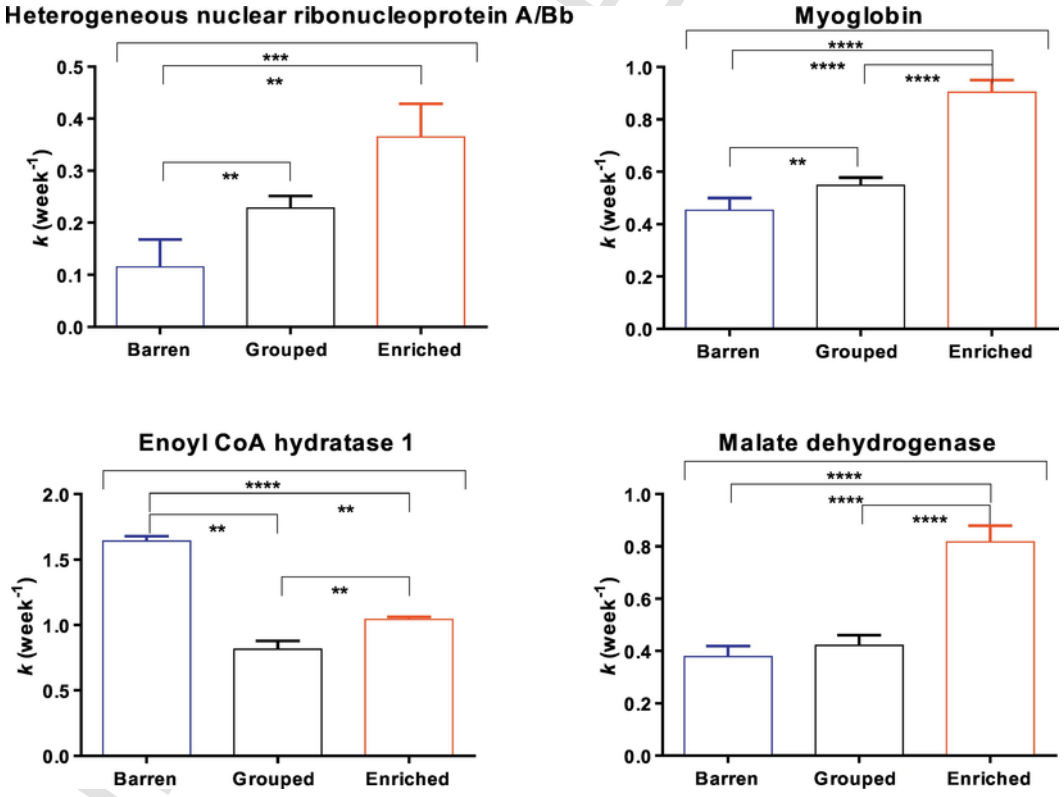

Fig. 5. Effect of different stress conditions on the absolute rates of degradation of soluble cardiac proteins in zebrafish. The rates of degradation for individual proteins were compared across the experimental cohort. Exemplar plots of four proteins are shown. The significance of the difference between each condition is indicated. For heterogeneous nuclear ribonucleoprotein the significance was – all conditions $p = 0.0029$; barren v grouped $p = 0.0286$; barren v enriched $p = 0.0286$; grouped v enriched $p = 0.2$, for myoglobin - all conditions $p < 0.0001$; barren v grouped $p = 0.0265$; barren v enriched $p < 0.0001$; grouped v enriched $p < 0.0001$, for enoyl CoA hydratase - all conditions $p = 0.0002$; barren v grouped $p = 0.0286$; barren v enriched $p = 0.0286$; grouped v enriched $p = 0.0286$, for malate dehydrogenase - all conditions $p < 0.0001$; barren v grouped $p = 0.6684$; barren v enriched $p < 0.0001$; grouped v enriched $p < 0.0001$.

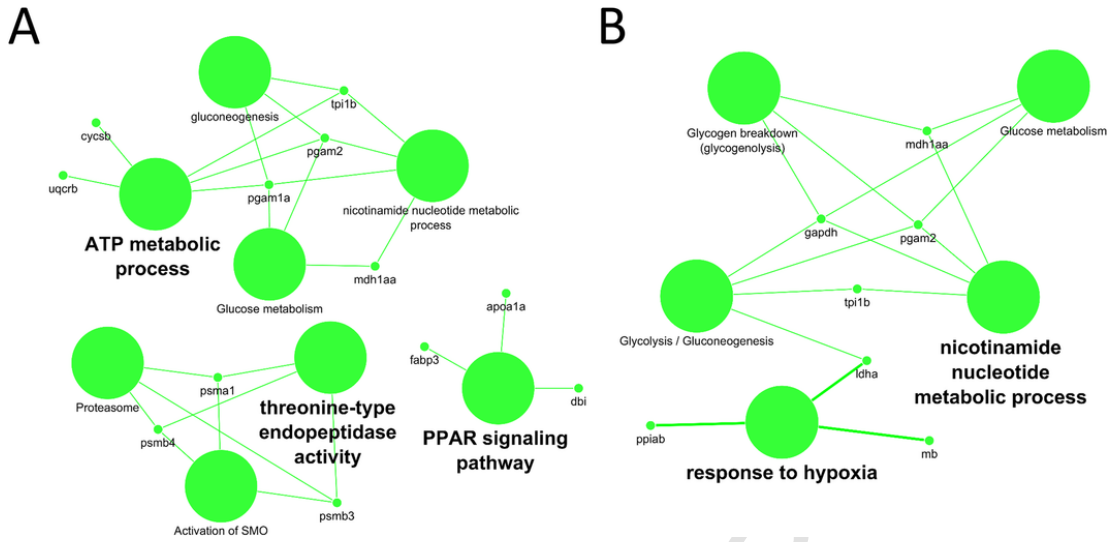

Fig. 6. Gene ontology mapping of soluble cardiac protein pathways. (A) Proteins with an increase in protein degradation in the enriched cohort were compared to the proteins increased in the barren group. GO analysis showed that proteins with an increase in protein degradation in the enriched group compared to the barren one clustered into three main functional groups. (B) Proteins from the enriched cohort with an increase in protein degradation were compared to the grouped zebrafish cohort. GO analysis revealed that molecules with an increase in protein degradation in the enriched group compared to the grouped zebrafish cohort could be clustered into two main functional groups. All proteins shown were specifically up-regulated in the enriched group. Statistical significance for all groups shown is ≤0.05 after correction for multiple testing.

4. Discussion

Changing the environment of zebrafish has previously been shown to alter their behaviour and induce a stress response [31]. Fish tend to prefer living in groups and with an enriched environment e.g. access to plant life. Fish housed individually with no enrichment show increased anxiety-like behaviours [32]. Fish housed in groups also show preference for specific types of enrichment [28]. In this study we have utilised well-established methods of isolation and decreased stimulation to model stress in the zebrafish. The measurement of cortisol in the tank water revealed that the zebrafish housed in a barren environment were more stressed than those fish maintained in the enriched environments. In addition, it is known from previous studies that fish maintained in a group but with no access to plant and gravel (the enrichment) will migrate to an enriched environment when possible [33]. Although the cortisol value for our grouped cohort could not be included in the statistical evaluation (n = 1), it did indicate that these fish were more stressed than those maintained in an enriched environment and less stressed than those kept individually in the barren environment.

We subsequently measured the rates of degradation of soluble cardiac muscle proteins from each cohort of zebrafish. At a global level, protein degradation was found to decline as the stress response increased. This indicates that the more stressed zebrafish were degrading proteins at a slower rate than the less stressed fish. At an individual protein level however, both increased and decreased protein degradation was observed between the different experimental groups. Four pathways were highlighted as having decreased degradation in the stressed phenotype compared to the non-stressed zebrafish. In both the barren and grouped conditions, there was a decrease in degradation of proteins involved in metabolic processes including glucose metabolism, glycogenolysis and gluconeogenesis and nicotinamide nucleotide metabolic processes. In the immediate stress response there is an activation of the neuroendocrine system. The subsequent release of stress hormones results in elevated blood glucose concentrations and stimulates the catabolic processes involved in the liberation of amino acids and other substrates for gluconeogenesis [15]. An increase in glucose concentration would be useful for the anticipated increase in energy demand, required by cardiac muscle as part of a fight or flight response. However it should be noted that not all proteins involved in energy production show a decreased degradation under stressed conditions. This includes enoyl CoA hydratase, which catalyses the second step of β-oxidation showed an increased rate of protein degradation in fish maintained under barren conditions. This may reflect the utilisation of different pathways for energy generation under stressed conditions, with a preference for glucose as a resource over fatty acids.

A further functional grouping that showed statistically significant changes in protein degradation in response to stress was proteins associated with PPAR signalling. Again, the rate of protein degradation was found to be lower in zebrafish with the most stressed phenotype. PPAR proteins are a nuclear hormone receptor superfamily of ligand-activated transcription factors [34,35]. Previous studies have implicated both PPARα and PPARγ as having a potential role in regulating the stress response [36]. Exposure to stress has been found to increase expression and activity of PPARγ in the brain that may affect acute neuronal responses [37,38]. PPARα regulates genes involved in lipid metabolism with a defined role in normal cardiac metabolic homeostasis. It controls the expression of enzymes involved in fatty acid β-oxidation and has been identified as participating in cardiac p38 kinase-dependent stress-activated signalling [39]. PPARα is proposed to act as a link between extracellular stressors and alterations in energy metabolism [40]. This would reflect the required shift in metabolism under stressed conditions.

There was also a decrease in degradation rate of proteins related to the hypoxic responses. A tolerance to hypoxia is a well-defined stress indicator and by maintaining these proteins, the zebrafish would be in a more favourable position to respond to stressor such as predators. The UPS has also been implicated in the response to hypoxia and may act as a co-ordinator of the stress response [18]. In the zebrafish that exhibited the most extreme stress phenotype, components of the UPS showed a decreased level of protein degradation suggesting that in a stressed state the cell acts to maintain control and integrity of the system. Proteolysis by the UPS plays an important role in stress response pathways by rapidly degrading any damaged or unwanted proteins and recycling amino acids for new protein synthesis. Once the stress has been detected, cells transduce the signal to ensure that the appropriate countermeasures are switched-on. The stress signal ultimately reaches

transcription factors to activate a stress response by modulation of gene expression. The subsequent increase in repair and defence capacities may be sufficient for adaptation to the stress condition.

5. Conclusions

Stressful events elicit a multitude of biochemical changes, which makes it possible to describe the stress reaction in molecular terms. Protein degradation is a major tool that an organism can use to maintain cellular homeostasis during times of stress. In this study we induced a stress phenotype in zebrafish by manipulating their local environment and ability to interact with other fish. The rate of degradation of soluble cardiac muscle proteins under each stress condition was measured using stable isotope labelling in conjunction with high resolution mass spectrometric analysis. It was observed that the greater the stress response in the zebrafish the lower the level of overall protein degradation. This suggests that during stress the cell may reduce the basal degradation of proteins and protect specific pathways important to survival. In doing so, the organism conserves both energy and resource to use in a "danger" situation.

Detailed GO analysis revealed four primary pathways with a decrease in individual protein degradation during the stressed phenotype. These have all been previously correlated with the stress response be that as part of a 'fight or flight' response or in the regulation pathways linked to stress amelioration. By focussing specifically on the heart, an organ that is often linked to stress-induced dysfunction we have provided additional information into the cardiac stress response at a molecular level. In future work, we intend to further explore these pathways in cellular systems and higher organisms to determine the specific role that protein turnover has to play in mediating the stress response.

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The degradation rates of 287 proteins were calculated from two time-points with at least two unique peptides used in the identification (Supplemental Table 1). ClueGO analysis grouped the proteins into fifteen functional families - actin filament depolymerisation, cellular response to oxidative stress, energy derivation by oxidation of organic compounds, gas transport, generation of precursor metabolites and energy, glutathione transferase activity, intermediate filament, one-carbon metabolic process, oxidation-reduction process, oxidoreductase activity- acting on the aldehyde or oxo group of donors, purine ribonucleoside triphosphate metabolic process, response to hypoxia, single-organism catabolic process, small molecule metabolic process and threonine-type endopeptidase activity (Supplemental Fig. 1). This broad range of ontologies is representative of the functions of the soluble cardiac muscle proteome.